IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Regulator of Calcineurin 3 Ameliorates Autoimmune Arthritis by Suppressing Th17 Cell Differentiation

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Regulator of calcineurin 3 (RCAN3), an endogenous regulator of the calcineurin–nuclear factor of activated T cells (NFAT) signaling pathway, inhibits the phosphatase activity of calcineurin, the nuclear translocation of NFAT, and the NFAT downstream pathway. To investigate the effects of RCAN3 on T-cell regulatory function and the development and progression of inflammatory arthritis, we studied the effects of RCAN3 transfection on regulation of Th17 cell differentiation in a murine T-lymphoma cell line and primary splenic CD4⁺ T cells. Overexpression of RCAN3 suppressed Th17 cell differentiation through the down-regulation of RAR receptor orphan receptor γ T mRNA and up-regulation of forkhead box P3 mRNA. In mice with collagen-induced arthritis, injection of an RCAN3-overexpression vector controlled arthritis development *in vivo*. Injection of RCAN3 reduced the formation of osteoclasts and expression of inflammatory cytokines *in vivo*. Antioxidants stimulated the expression of RCAN3 *in vitro*, and combination therapy with pcDNA-RCAN3 had a synergistic suppressive effect on the development of arthritis. These data suggest that RCAN3 may be an effective treatment for rheumatoid arthritis. (*Am J Pathol* 2017, 187: 2034–2045; <http://dx.doi.org/10.1016/j.ajpath.2017.05.008>)

Rheumatoid arthritis (RA) is a systemic autoimmune disease that can cause joint erosion and osteoporosis. Its pathogenesis is characterized by hyperplasia of synovial tissue and progressive destruction of articular cartilage structure.^{1,2} The pathogenesis of RA is a complex process that is mediated by multiple cytokines, proteolytic enzymes, and prostanoids. The expression of adhesion molecules and chemokines on synovial tissues increases the influx of immune cells, including T and B cells, which leads to induction of various inflammatory cytokines, such as tumor necrosis factor (TNF)- α , IL-1 β , IL-6, and IL-17A.³

CD4⁺ T cells are critically implicated in the pathogenesis of RA. Among the various effector T-cell subsets, Th17 cells, which are characterized by the production of their signature cytokine IL-17A, are a recently identified CD4⁺ T-cell subset linked to the induction of numerous

autoimmune diseases, including RA, multiple sclerosis, inflammatory bowel disease, and systemic lupus erythematosus.^{4–7} Another major subset of T cells, regulatory T (Treg) cells, play an opposing role to Th17 cells by controlling an excessive inflammatory response.⁸

The regulators of calcineurin (RCANs) form a functional subfamily with three members, RCAN1, RCAN2, and RCAN3, and are endogenous regulators of the

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calcineurin—nuclear factor of activated T cells (NFAT) signaling pathway.⁹ RCAN proteins have a highly conserved calyculin inhibitor of calcineurin motif, and RCAN competes with NFAT cytoplasmic 1 (NFATc1) for binding to calcineurin.^{10–12} RCAN inhibits the phosphatase activity of calcineurin, the nuclear translocation of NFAT, and the NFAT downstream pathway.^{13,14} RCAN is a potent regulator of several diseases, including Down syndrome, cardiac hypertrophy, Alzheimer disease, and breast cancer.^{15–18} However, it is unknown whether RCAN has therapeutic potential in autoimmune disease, especially in RA.

In this study, we investigated whether RCAN3, a calcineurin inhibitor, modulates arthritis in an animal arthritis model. Fortification of RCAN3 inhibited the differentiation of Th17 cells in a murine T-lymphoma cell line and splenic CD4⁺ T cells *in vitro*. Administration of pcDNA-RCAN3 reduced the incidence and severity of arthritis through inhibition of Th17 cells, related inflammatory cytokines, and osteoclast differentiation.

Materials and Methods

Animals

Four- to six-week-old male DBA/1J and BALB/c mice were purchased from Orient Bio Inc. (Seongnam, Korea). IL-1 receptor antagonist knockout mice were gifted from Professor Yoichiro Iwakura (University of Tokyo, Tokyo, Japan). SKG mice with a BALB/c background were gifted from Professor Shimon Sakaguchi (Osaka University, Osaka, Japan). Animals were maintained under specific pathogen-free conditions at the Institute of Medical Science of the Catholic University of Korea and were fed standard mouse chow and water. All experimental procedures were examined and approved by the Animal Research Ethics Committee of the Catholic University of Korea; the procedure conformed to all US National Institutes of Health guidelines.¹⁹

Arthritis Induction and Injection of Agents

Type II collagen was dissolved overnight in 0.1 N acetic acid (4 mg/mL) with gentle rotation at 4°C. DBA/1J mice were injected intradermally at the base of the tail with 100 µg of type II collagen emulsified in complete Freund adjuvant (1:1 w/v; Chondrex Inc., Redmond, WA). Two weeks later, 100 µg of type II collagen was dissolved and emulsified 1:1 with incomplete Freund adjuvant (Difco, Franklin Lakes, NJ) and injected into the hind leg as a booster injection. For induction of arthritis in SKG mice, zymosan A (Sigma-Aldrich, St. Louis, MO) was suspended in phosphate-buffered saline and incubated for 10 minutes in boiling water, and the zymosan A solution (2 mg per mouse) was then injected i.p. into 7- or 8-week-old mice. On day 7 after immunization, mice were injected i.v. with 100 µg of

pcDNA-RCAN3 in a large volume of saline (2 mL) within 5 seconds. After 8 days, the same mice were injected i.m. with 100 µg of the cDNA-RCAN3 in the left leg with electrical stimulation (electroporation). The i.m. injection was performed using a 31-gauge needle and insulin syringe for hydrodynamic-based procedures. Two days later, the mice were injected i.m. with 100 µg of pcDNA-RCAN3 in the right leg with electroporation. To assess the synergistic effects of the antioxidant grapeseed proanthocyanidin extract (GSPE; Hanlim Pharmaceutical Company, Seoul, Republic of Korea) on the severity of arthritis in the SKG mice, on day 7 after the first immunization, the mice were injected i.p. with 100 mg/kg of GSPE in saline or saline alone three times per week for the experimental periods.

Clinical Assessment of Arthritis

The severity of arthritis was determined by three independent observers (J.-H.J., J.-K.B., and S.-M.K.). To assess the severity of joint inflammation, the arthritis index was scored twice weekly from the onset of arthritis for up to 8 weeks after the primary immunization. The severity of arthritis was assessed on a scale of 0 to 4 according to the following criteria, as described previously²⁰: 0, no edema or swelling; 1, slight edema and erythema limited to the foot or ankle; 2, slight edema and erythema from the ankle to the tarsal bone; 3, moderate edema and erythema from the ankle to the tarsal bone; and 4, edema and erythema from the ankle to the entire leg. The arthritic score for each mouse was expressed as the sum of the scores of four limbs. The highest possible arthritis score for a mouse was thus 16. The mean arthritis index was used to compare the control and experimental groups. Clinical scores in SKG mice were monitored weekly according to the following criteria: 0, no swelling or redness; 0.1, swelling or redness of digits; 0.5, mild swelling and/or redness of wrists or ankle joint; and 1, severe swelling of larger joints. The scores for the affected joints were totaled for each mouse.

Histopathologic Analysis

Joint tissues were fixed in 4% (v/v) paraformaldehyde, decalcified in a histologic decalcifying agent (Calci-Clear Rapid; National Diagnostics, Atlanta, GA), embedded in paraffin, and cut into sections. The sections (7 µm) were stained with hematoxylin and eosin and Safranin O. The extent of inflammation was scored as described previously.²¹ Inflammation was scored using the following criteria: 0, no inflammation; 1, slight thickening of the lining or infiltration of some cells into the underlying layer; 2, slight thickening of the lining with infiltration of some cells into the underlying layer; 3, thickening of the lining, with an influx of cells into the underlying layer and cells evident in the synovial space; and 4, extensive infiltration of the synovium by inflammatory cells. Cartilage damage was evaluated by staining with Safranin O and toluidine blue,

Table 1 Primers for PCR

Primer name	Forward primer	Reverse primer
IL-17A	5'-CCTCAAAGCTCAGCGTGTC-3'	5'-GAGCTCACTTTTGCGCCAAG-3'
TNF- α	5'-GATCGGTCCCCAAGGGATG-3'	5'-TGAGGGTCTGGGCCATAGAA-3'
IL-21	5'-AAGATTCTTGAGGATCCGAGAAG-3'	5'-GCATTCTGTAGCGTCTATAGTGTC-3'
Foxp3	5'-GGCCCTTCTCCAGGACAGA-3'	5'-GCTGATCATGGCTGGGTTGT-3'
ROR γ T	5'-TGTCTGGGCTACCCCTACTG-3'	5'-GTGCAGGAGTAGGCCACATT-3'
NFATc1	5'-CGGGAAGAAGATGGTGCTGT-3'	5'-TTGGACGGGGCTGGTATA-3'
Cathepsin K	5'-CAGCAGAGGTGTGTACTATG-3'	5'-GCGTTGTTCTTATTCCGAGC-3'
MMP9	5'-CTGTCCAGACCAAGGGTACAGCCT-3'	5'-GAGGTATAGTGGGACACATAGTGG-3'
Carbonic anhydrase II	5'-TGGTTCACCTGGAACACCAAA-3'	5'-AGCAAGGGTCGAAGTTAGCA-3'
Integrin β 3	5'-CCACACGAGGCGTGAAC-3'	5'-CTTCAGGTTACATCGGGGTGA-3'
RCAN3	5'-TGCCAGTGATCAACTATGACC-3'	5'-TCTTCCTCTTCAGTTTCGCTC-3'
β -actin	5'-GTACGACCAGAGGCATACAGG-3'	5'-GATGACGATATCGCTGCGCTG-3'

Foxp3, forkhead box P3; MMP9, matrix metalloproteinase 9; NFATc1, nuclear factor of activated T cells cytoplasmic 1; RCAN3, regulator of calcineurin 3; ROR γ T, retinoid orphan receptor γ T; TNF- α , tumor necrosis factor- α .

and the extent of damage was scored using the following criteria: 0, no destruction; 1, minimal erosion (limited to single spots); 2, slight-to-moderate erosion in a limited area; 3, more extensive erosion; and 4, general destruction.

Immunohistochemistry

Tissues were incubated with primary antibodies against TNF- α , IL-6, IL-17, vascular endothelial growth factor, NFATc1, and p53 (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C. Tissues were then incubated with a biotinylated secondary antibody, followed by incubation for 1 hour with streptavidin-peroxidase complex. The final colored products were developed using the chromogen diaminobenzidine (Thermo Fisher Scientific, Rockford, IL), and the sections were examined under a photomicroscope (Olympus, Tokyo, Japan). Positive cells were enumerated visually by four individuals, and the mean values were calculated.

Mixed Lymphocyte Reaction

Single-cell suspensions were prepared from the spleens and cultured at 2×10^5 cells per well in 96-well plates for 72 hours. Cultured cells were pulsed with 2.5 μ Ci/mL of 3 H-thymidine (GE Healthcare, Little Chalfont, England) during the last 16 hours of incubation. The incorporation of 3 H-thymidine was analyzed using a Betaplate scintillation counter (PerkinElmer, Wellesley, MA).

Isolation of Splenocytes and Splenic CD4⁺ T Cells and T-Cell Stimulation

Mouse spleens were sieved through a mesh, and red blood cells were lysed using hypotonic ACK buffer (0.15 mmol/L NH₄Cl, 1 mmol/L KCO₃, and 0.1 mmol/L EDTA, pH 7.4). The remaining splenocytes were maintained in RPMI 1640 medium that contained 5% fetal bovine serum. To purify splenic CD4⁺ T cells, the splenocytes were incubated with

CD4-coated magnetic beads and isolated on MACS separation columns (MiltenyiBiotec, Bergisch Gladbach, Germany). Cells were cultured in the presence of 0.5 μ g/mL of plate-bound anti-CD3 and 1 μ g/mL of soluble anti-CD28 (BD Biosciences, San Jose, CA) with anti-interferon (IFN)- γ (R&D Systems, Minneapolis, MN) and anti-IL-4 (R&D Systems) (each at a concentration of 10 μ g/mL) for 3 days. Th0 cells were stimulated only with anti-CD3 and anti-CD28 without added cytokines. Th17 differentiation was induced by treatment with 20 ng/mL of IL-6 (R&D Systems) and 2 ng/mL of transforming growth factor- β (R&D Systems).

Transfection

Transfection of pcDNA-RCAN3 constructs was performed using the 4D-nucleofector X unit (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer recommendations with program DN-100 (Lonza, Basel, Switzerland). Briefly, CD4⁺ cells or LBRM-33 murine T-cell lymphoma cells (clone 4A2, TIB155; ATCC, Manassas, VA) were stimulated with 0.5 μ g/mL of anti-CD3 for 12 hours, harvested, and transfected with the pcDNA-RCAN3 vector or mock vector using a T-Cell Nucleofector Kit (Amaxa Biosystems). Transfected cells were incubated in anti-CD3-coated plates for 4 hours and treated with anti-IFN- γ and anti-IL-4 for 2 hours, after which the cells were cultured under the required polarizing condition for 48 hours.

Intracellular Staining and Flow Cytometry

Splenocytes were immunostained with various combinations of the following fluorescence-conjugated antibodies: CD4, CD19, CD23, B220, IgD (eBioscience, San Diego, CA), IgM, CD21/CD35, CD138, GL-7 (BD Biosciences, San Diego, CA), and CD25 (BioLegend, San Diego, CA). These cells were also stained intracellularly with the following antibodies: IFN- γ (BioLegend), IL-4, IL-17, and forkhead box P3 (Foxp3) (eBioscience). Before intracellular

Figure 1 Regulator of calcineurin 3 (RCAN3) suppresses Th17 cell differentiation *in vitro*. **A** and **B**: LBRM-33 cells were transfected with the mock vector or pcDNA-RCAN3 vector and incubated for 2 days under the Th17 cell–polarizing condition. **A**: mRNA expression of RCAN3, retinoid orphan receptor γ T (ROR γ T), IL-17, and forkhead box P3 (FoxP3) was analyzed by real-time PCR. **B**: These cells were subjected to immunostaining to identify CD4⁺RCAN3⁺IL-17⁺ cells and CD4⁺CD25⁺Foxp3⁺ cells and analyzed by confocal microscopy. **C** and **D**: CD4⁺ T cells were isolated from the spleens of C57BL/6 mice and stimulated with anti-CD3 (0.5 μ g/mL for 12 hours). Cells were then transfected with the mock or pcDNA-RCAN3 vector and incubated for 2 days under the Th17 cell–polarizing condition. **C**: Expression of RCAN3, ROR γ T, IL-17, and Foxp3 was analyzed by real-time PCR. **D**: Tumor necrosis factor- α (TNF- α), IL-17, and IL-21 concentrations in cell supernatants was measured by enzyme-linked immunosorbent assay. Data are expressed as means $\pm$ SD. * P < 0.05, ** P < 0.01, and *** P < 0.001 versus mock vector group.

staining, the cells were stimulated with 25 ng/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich) and 250 ng/mL of ionomycin in the presence of GolgiStop (BD Biosciences) for 4 hours. Intracellular staining was performed using an intracellular staining kit (eBioscience) according to the manufacturer protocol. Events were collected using a FACSCalibur flow cytometer (BD Biosciences), and the data were analyzed using FlowJo software version 7.6 (Tree Star, Ashland, OR).

Confocal Microscopy of Immunostaining

Spleen tissues were snap frozen in liquid nitrogen and stored at -70°C . Spleen tissue sections (7 μm) were fixed in acetone, and Treg cells were stained using anti-CD4, anti-CD25, and anti-Foxp3 antibody. To stain Th1, Th2, and Th17 cells, anti-CD4, anti-IFN- γ , anti-IL-4, and anti-IL-17A antibodies were used. The sections were incubated overnight at 4°C and stained, and the staining was analyzed using an LSM 510 Meta confocal microscopy system (Carl Zeiss, Oberkochen, Germany). Positive cells were counted visually at higher magnification by four individuals.

Real-Time PCR

A LightCycler 2.0 instrument (software version 4.0) (Roche Diagnostics, Indianapolis, IN) was used for PCR amplification. All reactions were performed with LightCyclerFastStart DNA Master SYBR Green I (Takara, Tokyo, Japan) according to the manufacturer instructions. Primer sequences are shown in Table 1. The level of mRNA expression was normalized to that of β -actin mRNA.

ELISA

The amounts of IL-17, IL-21, and TNF- α in the culture supernatants from the mouse cells were measured by sandwich enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Horseradish peroxidase–avidin (R&D Systems) was used for color development. Absorbance was measured at 405 nm on an ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

Measurement of Immunoglobulin Concentrations

Blood from the orbital sinus of mice was obtained, and serum samples were stored at -20°C until use. The amounts of antibody to total IgG, IgG1, and IgG2a in the blood or supernatants from cultured splenocytes were measured using mouse IgG, IgG1, and IgG2a ELISA quantification kits (Bethyl Laboratories, Montgomery, TX), respectively. The absorbance values were measured at 450 nm on an ELISA microplate reader.

Osteoclast Differentiation

Bone marrow cells were isolated from the tibias and femurs of mice by flushing the bone marrow cavity with α –minimum essential medium (Invitrogen, Carlsbad, CA). The cells were incubated with α –minimum essential medium that contained 10% fetal bovine serum for 12 hours to separate the floating and adherent cells. The floating cells were seeded in 48-well plates at 2×10^5 cells per well and cultured with α –minimum essential medium in the presence

Figure 2 Systemic delivery of the pcDNA-regulator of calcineurin 3 (RCAN3) vector suppresses the development of arthritis. **A:** Eight days after collagen-induced arthritis (CIA) induction, the pcDNA-RCAN3 vector was administered by hydrodynamic i.v. injection. After 8 and 10 days, mice received two additional injections of the RCAN3 vector into the muscles of both thighs by electroporation (red arrows). Arthritis development was assessed by measuring the arthritis score and incidence. **B:** Sections of articular tissue were prepared from mice treated as described in **A** 80 days after CIA induction and stained with hematoxylin and eosin (H&E), Safranin O, and tartrate-resistant acid phosphatase (TRAP). Arrows indicate the TRAP-positive cells. Representative histologic features are shown. The graphs depict the quantified degree of inflammation. **C:** The serum concentrations of IgG, IgG1, and IgG2a were measured by enzyme-linked immunosorbent assay. **D:** Mice were sacrificed 80 days after CIA induction, and isolated splenocytes were cultured *in vitro* for 3 days. Incorporation of $[^3H]$ thymidine was measured. **E:** Bone marrow cells were isolated from mice treated with the pcDNA-RCAN3 or control vector 80 days after CIA induction, and osteoclast precursor cells were induced by incubation with macrophage colony-stimulating factor (M-CSF). The cells were cultured with 25 ng/mL of M-CSF and receptor activator of NF- κ B ligand for 4 days and stained for TRAP activity. Multinucleated TRAP $^{+}$ cells were counted as osteoclasts. **F:** RNA was extracted from differentiated cells described in **E** and the expression of nuclear factor of activated T cells cytoplasmic 1 (NFATc1), cathepsin K, matrix metalloproteinase 9 (MMP9), carbonic anhydrase II (CAII), and integrin β was analyzed by real-time PCR. Data are expressed as means $\pm$ SD. $n = 5$ per group (**A**). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus the pcDNA-RCAN3 vector group. Original magnification: $\times 40$ (**B**, first column); $\times 200$ (**B**, second and third columns). CPM, counts per minute.

of 10 ng/mL of recombinant human (rh) macrophage colony-stimulating factor (M-CSF) (R&D Systems) for 3 days to form macrophage-like osteoclast precursor cells. After 3 days, these osteoclast precursor cells were then cultured in the presence of 10 ng/mL of rh M-CSF and 25 ng/mL of soluble rh receptor activator of NF- κ B ligand (PeproTech, London, England) for 4 days to generate osteoclasts.

TRAP Staining

A commercial tartrate-resistant acid phosphatase (TRAP) kit (Sigma-Aldrich) was used according to the manufacturer

instructions but omitting counterstaining with hematoxylin. TRAP-positive cells containing three or more nuclei were scored as osteoclasts. TRAP-positive multinucleated cells were counted three times without knowledge of the previously counted numbers of osteoclasts.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 4 for Windows (GraphPad Software, Inc., San Diego, CA). P values were calculated using a two-tailed paired t -test and two-way analysis of variance (grouped). $P < 0.05$ was considered significant.

Figure 3 Regulator of calcineurin 3 (RCAN3) inhibits inflammatory cytokines through modulation of nuclear factor of activated T cells cytoplasmic 1 (NFATc1) and p53. **A–C:** Eight days after collagen-induced arthritis induction, the pcDNA-RCAN3 vector was administered by hydrodynamic i.v. injection. After 8 and 10 days, mice received two additional injections of the RCAN3 vector in the muscles of both thighs by electroporation. At 80 days after the first immunization with type II collagen, tissue sections were obtained from the ankle joints and stained with antibodies to tumor necrosis factor (TNF)- α , IL-6, and IL-17 (**A**), vascular endothelial growth factor (VEGF) (**B**), and the transcription factors NFATc1 and p53 (**C**). The micrographs show representative histologic features. Data are expressed as means $\pm$ SD number of antibody-positive cells. $n = 5$ animals per group. $**P < 0.01$, $***P < 0.001$ versus the pcDNA-RCAN3 vector group.

Results

RCAN3 Suppresses Th17 Cell Differentiation *in Vitro*

LBRM-33 T lymphoma cells were transfected with the RCAN3 vector to evaluate whether RCAN3 modulates the process of Th17 cell differentiation *in vitro*. Under the Th17 cell–polarizing condition, the mRNA expression of retinoid orphan receptor γ T (ROR γ T), a master transcription factor for Th17 cells, and IL-17 was suppressed, whereas the mRNA expression of Foxp3 was increased (Figure 1A). Under Th17 cell–polarizing condition, RCAN3 vector–transfected cells were less prone to differentiate toward CD4⁺IL-17⁺ cells and more prone to differentiate toward CD4⁺CD25⁺Foxp3⁺ cells than control cells transfected with the mock vector (Figure 1B).

To examine further the process of Th17 cell suppression observed in the T-lymphoma cell line, murine splenic CD4⁺ T cells were transfected with the RCAN3 vector, and results similar to those in T lymphoma cells were found. After stimulation under conditions that favored the development

of Th17 cells, the mRNA expression of ROR γ T and IL-17 decreased, and the mRNA expression of Foxp3 increased in the RCAN3-transfected condition than in the control condition (Figure 1C). The amounts of TNF- α and IL-17 were significantly lower in this condition than in the control condition (Figure 1D). These results suggest that RCAN3 regulates Th17 cells.

RCAN3 Modulates the Severity of Autoimmune Arthritis

To examine whether fortification of RCAN3 modulates the development and severity of arthritis *in vivo*, pcDNA-RCAN3 was administered to mice with collagen-induced arthritis (CIA). Injection of the pcDNA-RCAN3 vector into mice with CIA significantly suppressed the development of arthritis from the beginning, and this suppression was maintained for at least 80 days after the onset of arthritis (Figure 2A). Histologic examination of the joints stained with hematoxylin and eosin revealed less joint inflammation and cartilage destruction in mice injected with the pcDNA-RCAN3 vector compared with

Figure 4 Regulator of calcineurin 3 (RCAN3) controls the development of arthritis by suppressing Th17 cells *in vivo*. Splenocytes were isolated from mice treated with the pcDNA-RCAN3 vector or control vector 80 days after collagen-induced arthritis induction. **A:** The cells were stimulated with phorbol 12-myristate 13-acetate, ionomycin, and GolgiStop for 4 hours and then subjected to intracellular staining to identify interferon (IFN)- γ ⁺ Th1 cells, IL-17⁺ Th17 cells, IL-4⁺ Th2 cells, and CD25⁺forkhead box P3 (FoxP3)⁺ T-regulatory (Treg) cells among the CD4⁺ population. The proportions of Th1, Th2, Th17, and Treg cells were assessed by flow cytometry. **B and C:** Splenocytes were stained with antibodies against B220⁺IgD⁺IgM⁺ (T1) and B220⁺IgD^{high}IgM^{high} (T2) immature B cells, B220⁺IgD⁺IgM⁺ mature B cells, CD19⁺CD21⁺CD23⁺ marginal zone B (MZB) cells, CD19⁺CD21⁺CD23⁺ follicular B (FOB) cells, B220⁺GL-7⁺ germinal center (GC) B cells, and B220⁺CD138⁺ plasma cells (PCs) and analyzed by flow cytometry. Data are expressed as means $\pm$ SD. **P* < 0.05 versus the pcDNA-RCAN3 vector group.

the control vector-treated mice (Figure 2B). The levels of IgG, IgG1, and IgG2a were significantly lower in mice injected with the pcDNA-RCAN3 vector than in the control vector-treated mice (Figure 2C). T-cell proliferation was lower in mice injected with the pcDNA-RCAN3 vector than in the control vector-treated mice (Figure 2D).

The devastating bone destruction in RA occurs mainly because of the abnormal activation of osteoclasts, which are

responsible for bone resorption regulated by M-CSF and receptor activator of NF κ B ligand.²² To investigate the capacity of bone marrow cells from pcDNA-RCAN3- or control vector-injected mice with CIA, isolated bone marrow cells were induced to osteoclasts in the presence of M-CSF and receptor activator of NF κ B ligand. Fortification of RCAN3 in CIA mice markedly reduced osteoclast differentiation. These findings were evaluated by counting the number of TRAP⁺ cells per well (Figure 2E). To characterize the molecular mechanisms involved in osteoclast formation in the RCAN3-fortified condition, we used real-time PCR to measure the expression of osteoclastogenic markers, such as NFATc1, cathepsin K, matrix metalloproteinase 9, carbonic anhydrase II, and integrin β 3. The expression of these osteoclastogenic markers was lower in mice injected with the pcDNA-RCAN3 vector than in control vector-treated mice (Figure 2F). These results suggest that fortification of RCAN3 activity reduces the severity of arthritis.

RCAN3 Inhibits Inflammatory Cytokines through Modulation of NFATc1 and p53

To test whether RCAN3 has antiarthritic activity through the negative regulation of inflammatory mediators, joint sections were stained for inflammatory cytokines using immunohistochemistry (Figure 3). Control vector-treated mice had greater levels of IL-17, IL-6, and TNF- α in the joint tissue (Figure 3A). By contrast, the levels of these inflammatory mediators were lower in the joints from mice with excessive RCAN3 than in the joints from the control vector-treated mice. Vascular endothelial growth factor plays fundamental roles in angiogenesis and lymphangiogenesis, is a highly specific mitogen for endothelial cells, promotes cell migration, and inhibits apoptosis.²³ Fewer vascular endothelial growth factor-positive cells were observed in joint sections from mice treated with the RCAN3 vector than in sections from the control vector-treated mice (Figure 3B). To evaluate the effect of inhibition of calcineurin by RCAN3 in CIA, joint sections were stained for NFATc1 using immunohistochemistry. Overexpression of RCAN3 by the pcDNA vector caused suppression of NFATc1 expression in joint sections (Figure 3C).

The role of the p53 tumor suppressor in cancer is the focus of much research.²⁴ Previous observations have suggested that p53 may play a protective role in immune and inflammatory diseases by suppressing cytokines and increasing the destruction of pathogenic cells.^{25,26} More p53⁺ cells were found in the joint tissues from RCAN3-vector-treated mice than in joints from control mice (Figure 3C). These results implicate RCAN3 in the regulation of inflammatory cytokines through alterations in the expression of transcription factor, such as NFATc1 and p53.

Figure 5 Regulator of calcineurin 3 (RCAN3) acts through the reciprocal regulation of Th17 and T-regulatory (Treg) cells in mice with collagen-induced asthma (CIA). **A:** *Ex vivo* splenocytes were isolated from mice treated with the pcDNA-RCAN3 or control vector 80 days after CIA induction. RNA was extracted from the splenocytes and analyzed for the expression of IL-17, tumor necrosis factor (TNF)- α , IL-21, and forkhead box P3 (FoxP3) by real-time PCR. **B:** RNA was extracted from the splenocytes of *ex vivo* draining lymph node cells described in **A** 80 days after CIA induction and analyzed for the expression of IL-17, TNF- α , IL-21, and Foxp3 by real-time PCR. **C:** Spleens were isolated from mice treated with the pcDNA-RCAN3 or control vector 80 days after CIA induction and subjected to immunofluorescence staining to identify CD4⁺IL-17⁺ Th17 cells and CD4⁺CD25⁺Foxp3⁺ Treg cells. The numbers of Th17 cells and Treg cells were counted in four independent quadrants. Data are expressed as means $\pm$ SD. * P < 0.05, ** P < 0.01, and *** P < 0.001 versus the pcDNA-RCAN3 vector group.

RCAN3 Controls the Development of Arthritis by Inhibiting Th17 Cells *in Vivo*

To determine whether RCAN3 overexpression can affect the population of effector T cells, we used flow cytometry to count the number of Th1, Th2, Th17, and Treg cells in the spleens from mice treated with the pcDNA-RCAN3 or control vector. Fewer Th17 cells were found in spleens from mice with excessive RCAN3 compared with the control vector-treated mice. By contrast, the number of CD4⁺IFN- γ ⁺ Th1 cells, CD4⁺IL-4⁺ Th2 cells, and CD4⁺CD25⁺Foxp3⁺ Treg cells was similar in the spleens from mice treated with pcDNA-RCAN3 or the control vector (Figure 4A). Next, we investigated whether fortification of RCAN3 would affect *in vivo* B-cell development. No

significant differences were found between groups for B220⁺IgD⁺IgM⁺ (T1) and B220⁺IgD^{high}IgM^{high} (T2) immature B cells, B220⁺IgD⁺IgM⁺ mature B cell, CD19⁺CD21⁺CD23⁺ marginal zone B cells, CD19⁺CD21⁺CD23⁺ follicular B cells, B220⁺GL-7⁺ germinal center B cells, and B220⁺CD138⁺ plasma cells (Figure 4, B and C).

In RA, IL-17, which is mainly produced by Th17 cells, activates various pathogenic cells by activating proinflammatory mediators, such as IL-6, IL-21, and TNF- α .^{6,27} To examine whether fortification of RCAN3 can regulate the expression of Th17 cells, we evaluated the mRNA expression of Th17-related inflammatory cytokines in *ex vivo* splenocytes from mice treated with the pcDNA-RCAN3 or control vector. RCAN3 overexpression significantly attenuated the expression level of IL-17

Figure 6 Fortification of regulator of calcineurin 3 (RCAN3) ameliorates the development of rheumatoid arthritis. **A:** Splenocytes from SKG mice were pretreated with grapeseed proanthocyanidin extract (GSPE) for 2 hours and stimulated with anti-CD3 antibody for 15 hours. Expression of RCAN3 was analyzed by real-time PCR. **B:** Eight days after zymosan A immunization, the pcDNA-RCAN3 vector was administered by hydrodynamic i.v. injection. After 8 and 10 days, mice received two additional injections of the RCAN3 vector in the muscles of both thighs by electroporation. Eight days after zymosan A immunization, mice were treated with 100 mg/kg GSPE in saline or with vehicle alone via intraperitoneal injection three times a week. Arthritis development was assessed by calculating the arthritis score and incidence. **C:** Mice described in **B** were sacrificed 60 days after zymosan A immunization, and the serum concentrations of total IgG, IgG1, and IgG2a were measured by enzyme-linked immunosorbent assay. Data are expressed as means $\pm$ SD. $n = 3$ per group (**B**). * $P < 0.05$, *** $P < 0.001$ pcDNA-RCAN3 vector group versus the pcDNA-mock vector with GSPE group.

($P < 0.001$) and increased the Foxp3 mRNA level (Figure 5A). Similar tendencies were observed in the draining lymph nodes from mice treated with pcDNA-RCAN3 compared with control vector-treated mice (Figure 5B).

To investigate the role of RCAN3 in the reciprocal regulation of Th17 and Treg cells, we used confocal microscopy to count the number of CD4⁺IL-17⁺ cells in the spleens from mice treated with the pcDNA-RCAN3 or control vector. The pcDNA-RCAN3 vector-treated mice had a higher percentage of Th17 cells and a lower percentage of CD4⁺CD25⁺Foxp3⁺ Treg cells in *ex vivo* splenocytes compared with the control vector-treated CIA mice (Figure 5C). These data are consistent with the differences between groups in the number of Th17 and Treg cells observed by flow cytometry (Figure 4A). These results suggest that RCAN3 is negatively associated with Th17 cells in CIA mice.

RCAN3 Controls the Severity of Arthritis Synergistically with Antioxidants

In a previous study, we reported that natural GSPE, an antioxidant, promoted the expression of RCAN3 in CIA mice.²⁸ On the basis of this observation, we examined whether GSPE can increase the expression of RCAN3 in splenocytes from SKG mice, which are genetically prone to develop

autoimmune arthritis as a consequence of a point mutation in the SH2 domain of ZAP-70.²⁹ In the presence of anti-CD3 antibody, GSPE promoted the expression of RCAN3 in splenocytes from SKG mice (Figure 6A). To test the synergistic effects of fortification of RCAN3 and GSPE in RA, pcDNA-RCAN3 and GSPE were administered together to SKG mice. Zymosan A-injected mice, injected with both the pcDNA-RCAN3 vector and GSPE, had significantly lower arthritis scores compared with arthritic mice injected with the pcDNA-RCAN3 vector alone (Figure 6B). In addition, on day 60 after the first immunization, total IgG, IgG1, and IgG2a production was significantly lower in the mice treated with the pcDNA-RCAN3 vector and GSPE than in those treated with only the pcDNA-RCAN3 vector (Figure 6C). These results support the concept that RCAN3 is involved in the modulation of RA development.

Discussion

In the present study, we focused on determining whether RCAN3 plays a therapeutic role in autoimmune arthritis caused by pathogenic Th17 cells and osteoclasts. The *in vitro* and *in vivo* results indicated that RCAN3 could inhibit the differentiation of Th17 cells and expression of inflammatory cytokines. Fortification of RCAN3 reduces the severity of autoimmune arthritis by exerting

reciprocal regulation between Th17 cells and Treg cells, as well as by suppressing osteoclast differentiation. Antioxidants may trigger the expression of RCAN3 and may have a synergistic effect with RCAN3 in the treatment of RA.

The calcineurin inhibitors, such as cyclosporine and tacrolimus, are used as immunosuppressants for transplant therapy and as disease-modifying antirheumatic drugs when administered alone or in combination with other less specific drugs.^{30,31} These drugs require binding to cytosolic immunophilins and promote a sustained and ubiquitous inhibition of calcineurin phosphatase activity. Thus, despite their clinical efficacy, prolonged treatment with these calcineurin inhibitors can cause extensive adverse effects.³² Because of the concerns about calcineurin inhibitor toxicity, identifying new targets with greater specificity, less toxicity, and immunomodulatory effects is important for the development of strategies to control the progression of RA. Recently, it has been reported that RCAN can physically interact with calcineurin and modulate its phosphatase activity.³³ RCAN1 has two independent calcineurin-interacting motifs—CIC (calcineurin inhibitor of calcipressin) and PXIXXT—and RCAN3 has a CIC motif. The RCAN-derived peptide does not inhibit general calcineurin phosphatase activity, and only the RCAN CIC motif is responsible for inhibiting the calcineurin-NFAT-dependent signal pathway, which suggests that it has a specific immunosuppressive effect on the calcineurin-NFAT signaling pathway.^{14,34,35}

The role of RCAN protein as an endogenous modulator of calcineurin-NFATc signaling has gained attention recently. However, the effects of RCANs, especially RCAN3, in autoimmune diseases such as RA have not been determined. Therefore, it would be intriguing to identify whether RCAN3-mediated modulation of RA involves an immunomodulatory role. In this study, we found that fortification of RCAN3 suppressed Th17 cell development *in vivo* and *in vitro*. Signal transducer and activation of transcription 3 plays a critical role in the differentiation of CD4⁺ T cells into Th17 cells. It is directly involved in multiple major transcription factor genes, including the genes encoding ROR γ t, IFN regulatory factor 4, aryl hydrocarbon receptor, and c-Maf, which drive Th17 cell differentiation.^{36–38} NFATc1 was recently found to be important for the differentiation of Th17 cells via induction of ROR γ T. Deficiency of NFATc1 protects against the development of experimental autoimmune encephalomyelitis.³⁹

We have previously reported that p53 can alleviate autoimmune arthritis by controlling the balance between Th17 and Treg cells through direct binding to signal transducer and activation of transcription 3 and signal transducer and activation of transcription 5, which regulates Th17 and Treg cell differentiation.⁴⁰ In this study, we observed that the expression of NFATc1 was reduced but the expression of p53 was increased in joint tissues from pcDNA-

RCAN3-transfected mice with CIA compared with control mice with CIA. These results suggest that RCAN3 may link with NFATc1 and p53 in the immune network. Further studies are needed to define the nature of the associations among RCAN3, NFATc1, and p53.

Osteoclasts in the synovium play a significant role in RA-associated bone destruction,⁴¹ and the induction of osteoclastogenesis involves receptor activator of NF κ B ligand expression in synovial fibroblasts.⁴² Multinucleated giant osteoclasts have been observed at the site of RA bone erosion, called the synovium-bone interface, which implies their involvement in bone destruction.⁴³ NFATc1 is a master transcription factor for osteoclast differentiation.⁴⁴ We confirmed that the differentiation of bone marrow cells to mature TRAP⁺ osteoclasts was decreased in RCAN3-fortified mice with CIA compared with control mice with CIA. This finding suggests that RCAN3 may be directly involved in osteoclast differentiation, although further investigations are needed to understand the exact role of RCAN3 in osteoclastogenesis.

In conclusion, our data are the first to indicate that RCAN3 overexpression can improve the arthritis parameters of inflammatory arthritis. Fortification of RCAN3 suppressed the differentiation of Th17 cells *in vivo* and *in vitro* and reduced the formation of osteoclasts *in vivo*. Antioxidants may trigger the expression of RCAN3, and combination therapy with pcDNA-RCAN3 had synergistic suppressive effects on the development of arthritis. Our results suggest a potential use of RCAN3 as a novel therapeutic target in autoimmune arthritis.

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